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Changes in subcellular localization of fructose 1,6-bisphosphatase during differentiation of isolated muscle satellite cells

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Abstract **Subcellular localization of FBPase, a regulatory enzyme of glyconeogenesis, was examined inside dividing and differentiating satellite cells from rat muscle. In dividing myoblasts, FBPase was located in cytosol and nuclei. When divisions ceased, FBPase became restricted to the cytosolic compartment and finally was found to associate with the Z-lines, as in adult muscle. Moreover, a 12-fold decrease was observed in the number of FBPase-positive nuclei associated with muscle fibres of adult rat, as compared with young muscle, possibly reflecting the reduction in number of active satellite cells during muscle maturation. The data might suggest that FBPase participates in some nuclear processes during development and regeneration of skeletal muscle.**

Keywords: Muscle FBPase; Satellite cells; Myoblasts; Nuclei

1. Introduction

Muscle fructose 1,6-bisphosphatase (FBPase; EC 3.1.3.11) – a key enzyme of glycogen synthesis from non-carbohydrates [1–3] – for a long time was regarded to be soluble and freely diffused in the cytoplasm. However, our recent investigation revealed that, in striated muscles of mammals, FBPase co-localizes with sarcomeric α-actinin [4,5] and in cardiomyocytes and smooth muscle cells it is also present inside the cells' nuclei [6,7]. We have also demonstrated that FBPase transport to a cardiomyocyte nucleus requires the presence of cytosolic factors and proceeds through the nuclear pores [8].

A great number of enzymes, which have primarily been described as participating in the carbohydrates metabolism in the cytoplasm, have so far been found in the cells' nuclei. Among them, only glycogen synthase does not seem to participate in nuclear processes [9]. Other enzymes, such as glyceraldehyde 3-phosphate dehydrogenase, lactate dehydrogenase or phosphoglycerate kinase, are involved in DNA synthesis, transcription and reparation, and also in cell cycle progression [10–12]. Based on this finding, it is not unlikely that the same could apply in the case of FBPase, in that it might have a different physiological role in each of these subcellular compartments.

It is well known that smooth muscle cells retain their mitotic capacity during the whole life. Heart muscle cells were thought to lose the capability of mitotic divisions in the early stages of postnatal life, however, recent results of Kajstura et al. [13] challenged this prevailing opinion, demonstrating that a considerable amount of cardiomyocytes undergo mitosis in normal human hearts and that, in the ischemic hearts, the amount of dividing cells increase. The proliferative capability of adult cardiomyocytes was also demonstrated by Engel et al. [14]. By contrast, skeletal muscle fibres do not divide. The fact that they also lack nuclear FBPase encourages the speculation that FBPase in the nuclei might participate in DNA replication or cell cycle progression.

In skeletal muscle fibres there is a pool of myogenic cells necessary for adult muscle regeneration. These precursor cells, known as satellite cells, are mitotically quiescent in normal skeletal muscle, but in response to injury, stretching or to exercise they start to proliferate and fuse with existing fibres [15,16]. When dissociated from adult muscle and grown in culture the satellite cells are also able to proliferate, fuse and form multinucleate myotubes [16].

In order to test the assumption that muscle FBPase is involved in the regulation of some events during cell cycle, we examined the subcellular localization of FBPase in the dividing and differentiating satellite cells isolated from a rat soleus muscle. A physiological meaning of the achieved results is discussed.

2. Materials and methods

2.1. Chemicals

Dulbecco's modified eagle medium and fetal bovine serum were from Invitrogen (Breda, Netherlands). Other chemicals were obtained from Sigma (St. Louis, USA). All of the reagents were of the highest purity commercially available.

2.2. Antibody production

Mouse polyclonal antibodies against rabbit muscle FBPase were produced and purified as described previously [6]. To check specificity of the antibodies, immunoblotting was performed as described by Towbin et al. [17].

2.3. Isolation and primary culture of satellite cells

Soleus muscles were excised from the legs of 3-month-old, male Wistar rats. Satellite cells were isolated from the muscles by digestion with 0.15% pronase and cultured as previously described [18].

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Abbreviations: FBPase, fructose 1,6-bisphosphatase; FITC, fluorescein isothiocyanate; TRITC, tetramethyl-rhodamine isothiocyanate

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2.4. Light microscopy

Cultured cells, taken at different stages of myogenic development, were fixed in 3% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS, and blocked with 0.25% glycine and 3% bovine serum albumin. Immunostaining was carried out as described previously [6] and involved incubation of the cells with polyclonal anti-muscle FBPase antibodies, biotinylated secondary antibodies, and an extravidin peroxidase complex. In control reactions the primary antibody was omitted, or normal serum was used as a first layer. Specific recognition of FBPase by its antiserum was also checked using anti-FBPase serum pre-incubated with saturating concentrations of purified muscle FBPase for staining of satellite cells with the immunoperoxidase method.

2.5. Confocal microscopy

Cultured cells were treated as described for light microscopy. In a co-localization experiment, the cells were incubated with mouse anti-muscle FBPase polyclonal antibodies, with secondary, tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-mouse antibodies and subsequently with rabbit anti-MyoD or anti-myogenin antibodies and fluorescein isothiocyanate (FITC)-conjugates anti-rabbit antibodies. In control reactions, one of the primary antibodies was omitted.

In an experiment designed to examine the localization of FBPase in nuclei of neonatal and adult rat muscle, soleus muscles from 3-week-old and 9-month-old rats were excised, fixed immediately and embedded in polyester wax as described previously [4]. Longitudinal sections of 5-μm were cut, mounted on slides, de-waxed and stained with anti-FBPase antibodies and secondary FITC-conjugated antibodies. All nuclei were counterstained with propidium iodide. In each section, at least 200 nuclei were counted. The total number of nuclei was assessed first, then the number of FBPase-positive nuclei was recorded.

The fluorescent images were obtained with a Bio-Rad MRC 1024ES confocal scanner unit (equipped with a krypton/argon laser) and a Zeiss Axiovert S100 inverted microscope (with 40× or 63× Plan Apo Oil objective, NA = 1.4).

2.6. Activity measurement in isolated satellite cells

Satellite cells were isolated from rat soleus muscles as described above. The cells were pelleted by centrifugation ($140 \times g$, 10 min, $T = 4$ °C) and suspended in small volume of buffer: 50 mM Tris, 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride 1 mg/mL leupeptin; pH 7.4, $T = 4$ °C, with addition of 0.5% Triton X-100 to lyse the cells. The lysate was centrifuged at $5000 \times g$ for 10 min and supernatant was used to determine the FBPase activity.

FBPase activity was assayed in 50 mM bis–tris propane, 2 mM $MgCl_2$, 150 mM KCl, 1 mM EDTA, 0.2 mM NADP, 5 U/ml glucose 6-phosphate dehydrogenase, 2 U/ml glucose 6-phosphate isomerase, 50 μM fructose 1,6-bisphosphate, pH 7.5; the assay was carried out at 37 °C. The substrate was used to start the reaction. The measurements were performed with an HP 8452 A Diode Array Spectrophotometer.

3. Results

The cells used during the course of the study were obtained from three independent experiments.

3.1. Localization experiment

On day 3 after plating, FBPase was present both in the cytoplasm and in the nuclei, although predominantly in the nuclei of cells (Fig. 1A and C). Only in a small proportion (about 10%) of cells, the staining was confined to cytoplasm. Spindle-shaped, multinucleated myotubes appeared in culture on day 5 after plating. In such cells, the FBPase-positive staining became clearly restricted to the cytosol (Fig. 1B and D).

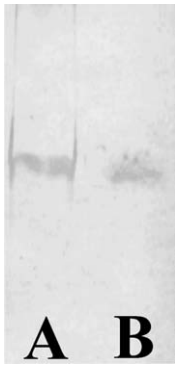

Fig. 2. Western blot of purified muscle FBPase (A) and satellite cells extract (B).

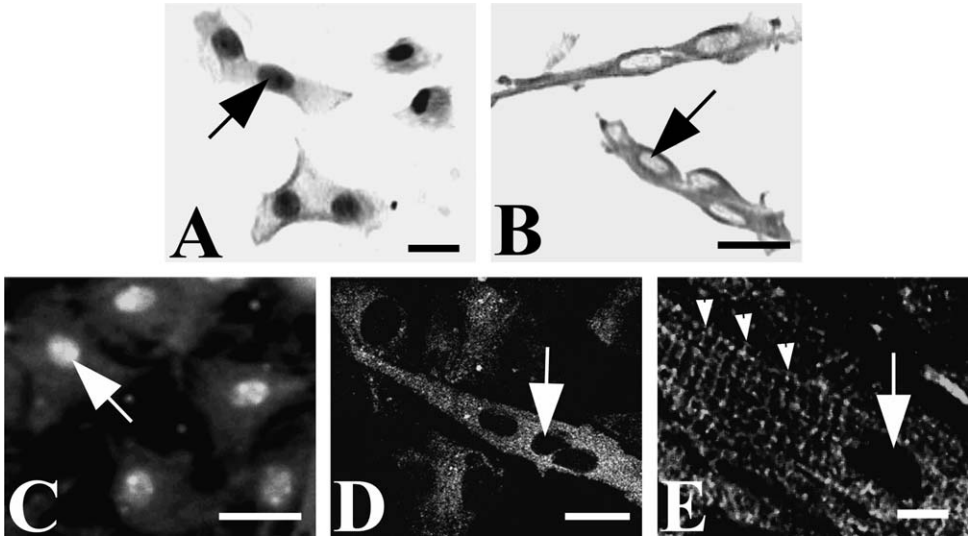

Fig. 1. Light and confocal microscopy study of FBPase localization in cultured satellite cells on day 3 (A, C), 5 (B, D) and 10 (E) after plating. Anti-FBPase IgGs were visualized with immunoperoxidase method (A, B) or with immunofluorescence using secondary, TRITC-conjugated antibodies (C, D, E). Arrows point to nuclei, arrowheads – Z-lines. Bar = 10 μm.

Finally, in mature myotubes with well-developed striation, FBPase accumulated at the Z-lines (Fig. 1E), as it was previously demonstrated for the skeletal muscles of adult mammals [4,5].

The use of anti-FBPase serum pre-incubated with purified muscle FBPase to the immunolocalization experiment resulted in lack of staining of the cells (data not shown).

3.2. Western blot

To check specificity of the interaction between the primary antibody and its antigen, a Western blot was performed. The results showed one band of 37 kDa in the satellite cells extract, corresponding to purified muscle FBPase, indicating that antibodies against muscle FBPase react with FBPase, but do not react with other proteins from rat satellite cells (Fig. 2).

3.3. Co-localization of FBPase and myogenic factors

Having identified two patterns of FBPase-positive reaction, double labeling of the cultured cells was performed to correlate the nuclear presence of FBPase with subsequent stages of satellite cells differentiation. In the experiment, we took advantage of antibodies against myogenic factors, MyoD or myogenin – the markers of respectively, proliferating and differentiating myoblasts [19]. These primary antibodies were subsequently detected with FITC-conjugated secondary antibodies. FBPase was visualized with TRITC-conjugated antibodies.

As a result, nuclear FBPase localization appeared to be confined to myoblasts expressing MyoD (Fig. 3A–C). In myogenin-positive cells FBPase seemed to be excluded from the nucleus and remained only in the cytoplasm (Fig. 3D–F). All controls were devoid of staining (data not shown).

3.4. Immunolabeling and counting of nuclear FBPase-positive satellite cells in young and mature rat muscle fibres

In the growing muscle, at least some of the satellite cells are mitotically active, contributing myonuclei to the enlarging fibres. By contrast, adult skeletal muscle contains very few, if any, dividing satellite cells [20]. But under stress conditions (injury or an increased workload), members of this self-renewing pool are activated again. Assuming that FBPase plays a role in the regulation of cell cycle progression, nuclear FBPase should be present in these activated satellite cells. In order to test this assumption, we have screened for the presence of FBPase-positive ($FBPase^+$) nuclei in the soleus muscle embedded in polyester wax, from a young (3-week-old) and adult (9-month-old) rat. In the experiment, FBPase was detected with FITC-conjugated antibodies and all nuclei within the sections were visualized with propidium iodide.

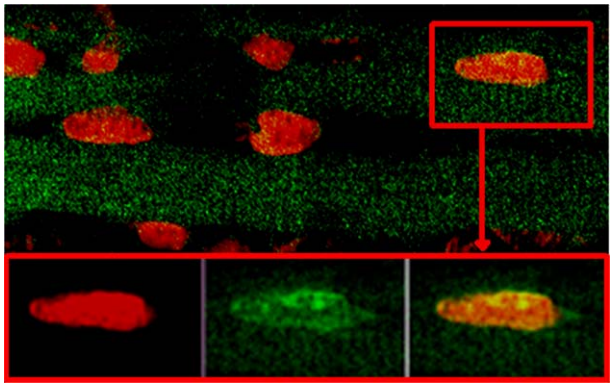

Fig. 4. Localization of FBPase in muscle fibres of adult rat. Anti-FBPase IgGs were detected with secondary, FITC-conjugated antibodies (green). Nuclei were contrasted with propidium iodide (red). FBPase-positive nucleus (enlarged in inset) might be attributed to the satellite cell activated by local stimuli before isolation.

In the examined sections of a young soleus muscle, FBPase was present only in a small subset of nuclei. The percentage of $FBPase^+$ nuclei was approximately 6%. In the adult muscle sections, the fibre-associated $FBPase^+$ nuclei were even fewer: only 0.5% were strongly stained with anti-FBPase antibodies (Fig. 4).

The satellite cells nuclei make up 12% of the nuclei associated with muscle fibres in a 36-day-old rat [21] and 2–7% in adult animals. A lower percentage of $FBPase^+$ nuclei than satellite cells associated with myofibres might suggest that the enzyme is located in the nuclei of activated satellite cells.

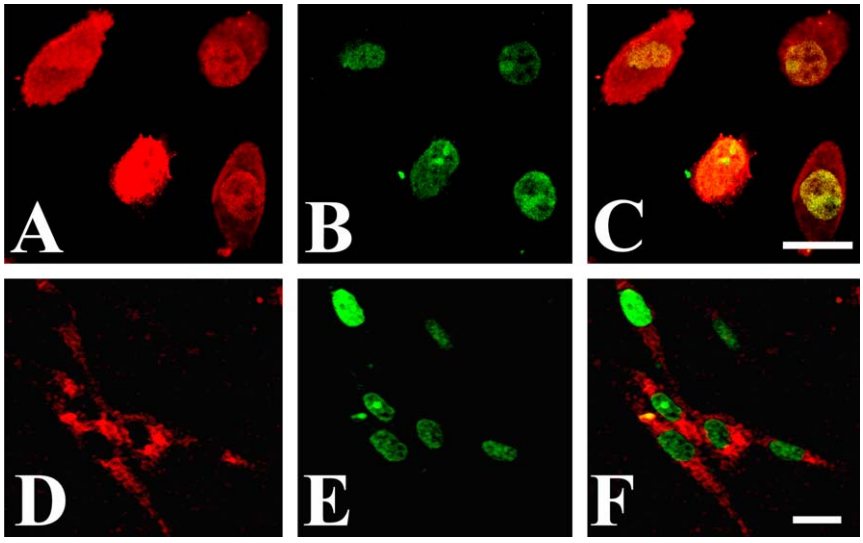

Fig. 3. Immunofluorescent localization of FBPase (red; A, C, D, F) in cultured satellite cells during the proliferative phase marked by MyoD expression (green; B, C) and during the differentiation phase, marked with the appearance of myogenin (green; E, F). Bar = 10 μm.

3.5. Activity measurement

Satellite cells isolated form a rat soleus muscle were counted in Thom-Zeiss cell and treated with 0.5% Triton X-100 to degrade the cells' membranes and to release the FBPase activity. Measured activity was 8 nU per one cell which corresponded to ca. 50 U per 1 g of the satellite cells.

4. Discussion

The aim of the present study was to characterize the subcellular localization of FBPase in the subsequent stages of development of activated muscle satellite cells.

Satellite cells isolated from rat skeletal muscles growing in culture are called myoblasts. Myoblasts adhere, proliferate and on day seven of culture they begin fusion into early 2–3-nuclei myotubes [18]. These events are under strict control of genes encoding two groups of the muscle-specific transcription factors, called the myogenic regulatory factors (MRFs) – the primary MRFs (MyoD and Myf-5) and the secondary MRFs (myogenin and MRF4) [19,22]. In studies of muscle regeneration, MyoD expression is routinely used as a marker of myoblast proliferation, and myogenin expression as a marker of the entry of cells onto the differentiation pathway. Using these markers, we demonstrated in the present study that the location of FBPase in the activated satellite cells was dependent on the phase of the cells. During the proliferative phase – marked by the expression of MyoD – FBPase was clearly present in the cytoplasm as well as in the cells nuclei. When the nuclei became myogenin-positive (which suggests that the differentiation phase began) the nuclear FBPase disappeared, and the positive reaction was restricted to the cytoplasm.

Comparing the number of $FBPase^+$ nuclei in muscles from young and adult rats we found that the percentage of such nuclei is approximately 12 times higher in a 3-week-old than in a 9-month-old rat. Since young rats grow rapidly, their satellite cells are believed to be proliferative [23], older rats grow slowly and their satellite cells are quiescent. Therefore, it is likely that $FBPase^+$ nuclei might be attributed to the satellite cells activated by local stimuli before isolation, and the observed decline in the number of $FBPase^+$ nuclei might reflect the reduction in number of active satellite cells which is associated with muscle maturation. The observed small percentage of $FBPase^+$ nuclei (0.5%) in a 9-month-old rat is consistent with the data of Schmalbruch and Lewis [24] suggesting that, in adult rat muscle, approximately 1–2% of myonuclei are replaced each week.

Information about the subcellular location of a protein and about its binding partners can provide valuable clues to the protein's role in a cell. An increasing body of evidence suggests that the association of FBPase with subcellular elements and other proteins (especially with aldolase and sarcomeric α-actinin) [4,25,26] plays an important role in the regulation of FBPase activity. In striated muscles, this association is inhibited by Ca^{2+} concentrations characteristic for contracting muscle [8,27], which indicates that binding of FBPase to cellular structures may play a role in controlling the speed of gluconeogenesis.

In the present paper, we demonstrate that FBPase is localized in nuclei of skeletal muscle precursors during their proliferative activity. This might indicate that, in addition to its 'classical' glyconeogenic role in cytoplasm, FBPase may participate in some nuclear processes during development and regeneration of skeletal muscle.

FBPase was also found in the nuclei of cardiac and smooth muscle cells – cells equally capable of dividing. However, the constant presence of FBPase in the nuclei of such cells might suggest a different nuclear role here to its role in dividing myoblasts.

Taken together, our findings might indicate that FBPase should be considered as another member of a growing family of "moonlighting" proteins [28] – proteins with more than one function depending on their cellular location. However, to identify the physiological role of FBPase in the nucleus, further studies are needed.

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